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Gestational and Hormonal effects on Magnesium Sulfate's Ability to Inhibit Mouse Uterine Contractility.

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Running head: Magnesium, gestation, oxytocin and uterine contractility.

Abstract

Magnesium sulfate is used as a tocolytic, but clinical efficacy has been seriously questioned. Our objective was to use controlled *ex vivo* conditions and known pregnancy stages, to investigate how two key factors, hormones and gestation, affect Magnesium's tocolytic ability. We hypothesized that these factors could underly the varying clinical findings around Magnesium's efficacy. Myometrial strips were obtained from non-pregnant (n=10), mid-term (n=12) and term-pregnant (n=11) mouse uterus. The strips were mounted in organ baths superfused with oxygenated physiological saline at pH 7.4 and 37 °C. The effect of different concentrations of $MgSO_4$ (2 – 20mM) was examined on spontaneous and oxytocin-induced (0.5-1nM) contractions. Contractile properties (amplitude, frequency and area under the curve, AUC) were measured before and after application of Magnesium. Magnesium sulfate had a dose-dependent inhibitory effect on both spontaneous and oxytocin-induced contractions but was less effective in the presence of oxytocin. In spontaneous contractions, Magnesium was more potent as gestation progressed ($P<0.0001$). In the presence of oxytocin however, there were no significant gestational differences in its effects on contraction. The rapid onset and reversal of Magnesium's effects suggest an extracellular action on Calcium entry. Taken together we conclude that Magnesium's actions are influenced by both gestational state and hormones, such that, at least in mice, it is least effective in early gestation with oxytocin present, and most effective at term in the absence of oxytocin. That Magnesium is least effective preterm and oxytocin decreases its effectiveness throughout gestation, may explain its disappointing clinical effects as a tocolytic.

Keywords: tocolysis, contraction, uterus, preterm birth. Oxytocin.

Introduction

The high incidence of preterm birth, >10% of births worldwide, remains unchanged despite much research effort and advancement in understanding uterine physiology [1]. It is the single largest cause of mortality and morbidity in newborns. Several tocolytics including oxytocin-receptor antagonists e.g. atosiban, prostaglandin synthesis inhibitors e.g. indomethacin, calcium channel blockers e.g. nifedipine, beta-2 agonists e.g. ritodrine, and magnesium sulfate ($MgSO_4$) have been developed to help prolong pregnancy, by reducing or slowing uterine contractions when preterm labour threatens. Unfortunately, it remains the case that none are ideal in terms of either efficacy or side effects, and perhaps not surprisingly there is little international consensus on which tocolytic to use to help manage spontaneous preterm labour.

Magnesium sulfate was described as a tocolytic in 1959 [2] and shortly after became the preferred drug in treating preterm labour. More recently however, studies including Cochrane systemic reviews[3] [4] have suggested that $MgSO_4$ is ineffective in the treatment of preterm labour. For example Crowther et al., (2014) concluded that it was no better than placebo for the primary outcome of giving birth within 48 hours of trial entry, and nor was there any significant difference for the primary outcome of serious infant morbidity [3]. In contrast, $MgSO_4$ has been shown to be the drug of choice in treatment of seizures in eclampsia[5] and prevention of preeclampsia in hypertensive pregnant women[6]. Additionally, $MgSO_4$ has been shown to have neuroprotective effects in preterm fetuses at delivery, reducing the risk of death, cerebral palsy, and gross motor dysfunction[7].

In contrast to clinical findings, *in vitro* studies have consistently found that Magnesium has a relaxant effect on smooth muscles including airway [8] and vascular [9]. Studies in myometrial smooth muscle show it to be a relaxant in several species[10-13]; it significantly inhibits contractions in a concentration-dependent manner[12,14].

In smooth muscles, Magnesium inhibits contractility via multiple mechanisms including effects on extracellular calcium entry, intracellular calcium release and calcium oscillations [15]. It primarily acts by competing with Ca^{2+} at the L-type, voltage-operated Calcium channel (VOCC), resulting in a decrease in intracellular calcium concentration [15] The channel is comprised of four subunits, of which α-1 is the pore forming, voltage sensitive and conducting component, and the others modulate its activity. Progression of gestation has been linked to an increase in α-1 expression, and VOCC activity increases close to term [16-18]. These findings suggest that Calcium channel density and expression increases with gestation. As Magnesium's main action is at the Calcium channel, the question arises: could these changes in Calcium channel density and expression alter the effectiveness of Magnesium at different gestational ages?

Of the hormonal changes around labour, oxytocin has a pivotal role [19]. Oxytocin stimulates myometrial activity via a variety of mechanisms, including increasing Ca^{2+} entry into the myometrium via L-type channels and stimulating Calcium release from the sarcoplasmic reticulum (SR)[20,21]. Oxytocin stimulation would therefore be expected to mitigate the actions of Magnesium. Thus if women with threatened preterm labour had differing levels of oxytocin or differences in levels of expression of its receptor[22], this may also alter responsiveness to Magnesium tocolysis *in vivo*.

It would be of benefit if the *in vivo* and *in vitro* findings concerning Magnesium's effects on uterine contractility could be reconciled. This could then enable a stratification of which threatened spontaneous preterm labors may benefit from its use as a tocolytic. Our approach was to consider if there may be physiological factors which affect Magnesium's efficacy *in vivo*. Preterm births are defined as those before 37 completed weeks of gestation, and thus cover a very large gestational age range and much change in myometrial physiology and hormonal conditions[23-26]. These changes can be anticipated to also affect Magnesium's tocolytic ability.

While the effect of Magnesium in animal and human term-pregnant myometrium on spontaneous [12-14] and oxytocin induced contractions [12,14,15], has received some attention, the effect of Magnesium at different gestational stages has not been systematically investigated. In addition, neither has the influence of oxytocin on Magnesium's action been studied throughout gestation. Furthermore, much can be learnt from mouse animal models, but we can find only one study where they have been used to look at Magnesium's relaxant effect, and that was on day 14 of pregnancy in lipopolysaccharide (LPS)-treated animals [27].

The aim of this study therefore was to investigate whether the relaxant effects of Magnesium are altered by gestational state and to test the hypothesis that the stimulation provided by oxytocin will significantly reduce the relaxant effects of Magnesium.

Methods

Solutions

All study solutions were prepared fresh at the start of each experiment by direct dissolving of $MgSO_4$ or $MgCl_2$ in buffered physiological saline solution (PSS) composed of (mM): NaCl 154, KCl 5.6, $MgSO_4$ 1.2, $CaCl_2$ 2, Glucose 8 and HEPES 10.2 [28]. Basal or control $MgSO_4$ therefore was 1.2mM. Oxytocin was prepared in distilled water and added to the PSS to give a final concentration of 0.5nM (pregnant tissues) or 1nM (non-pregnant tissues). A 1nM concentration of oxytocin was used for non-pregnant tissues to ensure a similar stimulation to that seen in pregnant tissues was achieved. The concentration of water did not exceed 0.01%. All chemicals were obtained from Sigma UK.

Tissue collection and preparation[19]

Non-pregnant, 14- and 16- day (referred to as mid-pregnant) and 18 day (term) pregnant mice were humanely killed using CO_2 anaesthesia and cervical dislocation, in accordance with UK Home Office regulations. All mice used were between 8-10 weeks old. The uterus was removed, cleaned of placentas and membranes (where applicable) and full-thickness (endometrium intact) myometrial strips (1 x 2 x 10 mm) were dissected along the longitudinal axis[21]. Using surgical thread, individual strips were mounted between a fixed support and a 10g isometric force transducer (World Precision Instruments, UK) within a 5ml tissue bath (Linton Instruments, UK) under a resting tension of 5mN and were continuously superfused with oxygenated (95% O2, 5% CO2) PSS at pH 7.4, at a rate of 5mL/min and maintained at 37^{o}C to mimic physiological conditions[29].

Experimental protocol

For spontaneous contractions, strips were allowed to equilibrate for 45-60 minutes until regular, frequent and equal amplitude contractions were observed. For oxytocin-induced contractions, strips achieved regular spontaneous activity as above, ahead of addition of oxytocin which remained in the superfusate throughout. The uterine strips were then exposed to increasing concentrations of $MgSO_4$ from 2 – 10 mM (spontaneous) and 2 – 12 mM (oxytocin-induced) for 15 minutes or $MgSO_4$ or $MgCl_2$ (10mM and 20mM), either in the presence or absence of oxytocin.

Analysis and statistics

Myometrial contractions were continuously recorded via the force transducer connected to a data acquisition system equipped with Labtrax software (World Precision Instruments, UK)[29]. For each recording, the amplitude, frequency and force integral (area under the curve, (AUC) of contraction were measured, both during the control period (contractions occurring in the ten minutes immediately preceding the first application of Magnesium) and during the final ten minutes of each step in the concentration-response period. Data were analysed using Origin Pro 2015 (Origin Lab Corporation, MA, USA) and are presented as percentage of control to reflect the effect of Magnesium, where the control period is taken as 100%. Values given are mean ± standard error of the mean (SEM) unless stated otherwise and were compared by ANOVA., *n* is the number of myometrial tissue strips, each one from a different animal, N.

Concentration-response curves for AUC were fitted to the logistic equation with the use of non-linear regression. The concentration at which $MgSO_4$ caused a 50% reduction (IC_{50}) in

overall contractile activity (AUC) was calculated. LogIC50s were compared by the extra sum of squares F test or ANOVA followed by Bonferroni post-hoc analysis.

All statistical analysis was carried out using Graphpad Prism 5, significance was taken as $P<0.05$. Summary of analyses performed:

1) Effect of $MgSO_4$ vs. $MgCl_2$ - compared using t tests.

2) Tables 1 and 2- ANOVA with Bonferroni post hoc correction.

3) Comparison of $LogIC_{50}$ values for Magnesium on spontaneous or oxytocin contractions between gestational groups – ANOVA with Bonferroni post hoc correction.

4) Comparison of $LogIC_{50}$ values for effect of magnesium on spontaneous vs. oxytocin simulated contractions at each gestation - Extra sum of squares F-test.

Results

Control data and effects of Magnesium on late-pregnant mouse myometrium.

As there were little or no data concerning the actions of Magnesium on mouse myometrium, initial experiments were performed to, determine that Magnesium affected contractility, that the effects of $MgSO_4$ were due to Magnesium and not the anion, and to obtain an indicative concentration for concentration-response curves.

As shown in Figures 1A and 1B, stable, control contractions from pregnant mouse myometrium could be obtained for several hours without decrement in spontaneously active (A) and oxytocin-stimulated (B) tissues (typical of 9 preparations). Figure 1 also shows typical traces obtained using either 10 mM and 20 mM $MgSO_4$, (1C) or, $MgCl_2$ (1D), from three paired experiments (different animals), on oxytocin-stimulated pregnant myometrium. No significant differences were found between the effect of 10mM $MgSO_4$ and $MgCl_2$: amplitude: 70 $\pm$ 14% and 77 $\pm$ 9%; AUC: 50 $\pm$ 7 and 58 $\pm$ 2%, respectively, nor were any significant differences found between $MgSO_4$ or $MgCl_2$ during spontaneous activity (data not shown). Based on these data, the remaining experiments were performed using $MgSO_4$ as it is the sulfated form which is used clinically, and the effects of different concentrations and gestation were examined.

Effects of Magnesium on spontaneous contractions at different gestational states.

The effects of the application of increasing concentrations of $MgSO_4$ in the superfusate bathing the spontaneously contracting myometrial strips were examined and compared at day 14, 16 and 18 of pregnancy, and non-pregnant. Concentration-dependent inhibitory

effects of Magnesium were found at each gestational state investigated as well as non-pregnant tissues. Representative isometric recordings are shown in Figure 2. For all preparations, there was a decrease in frequency of contractions followed by a reduction in contraction amplitude. The mean data for frequency, amplitude and AUC for spontaneous contractions for each group are given in Table 1. The data show that Magnesium reduces spontaneous activity of the myometrium in all tissue groups, with its effect becoming more marked (and significant) as term approaches: In non-pregnant and day 14-gestation tissues, contractions in the presence of 10mM $MgSO_4$ still persisted, with the AUC being 40- and 18% of control respectively (Figure 2A-C). In term-pregnant myometrium 10mM $MgSO_4$ further and significantly reduced the AUC to negligible values (6%, Figure 2D). The increased effects of Magnesium with gestation can also be appreciated from the fact that, in non-pregnant, day 14 and 16 tissues, contractions were abolished in just 1/22 preparations (on day 16), whereas at term, Magnesium abolished contractions in over half the tissues (6/11 preparations). In all preparations however, including those where contractions were abolished (e.g. Figure 2D), spontaneous contractions recovered to control values upon return to normal physiological saline ($MgSO_4$ 1.2mM).

Plotting AUC concentration-response curves and calculating the IC_{50} values for Magnesium confirmed the mean data findings. The order of potency for $MgSO_4$ on spontaneous contractions was term-pregnant>mid-pregnant>non-pregnant, (Figure 2E). The IC_{50} values fell from 7.06mM ± 0.32 (non-pregnant, n=10) to 4.75mM ± 0.21 (d 14, n=9) to 3.08mM ± 0.16 (term, n=11, $P<0.001$). There was no significant difference between the IC_{50} values for $MgSO_4$ at 14d and 16d gestation (4.75mM ± 0.21 and 5.50mM ± 0.26 respectively, $P>0.05$).

Effects of Magnesium on oxytocin-induced contractions at different gestational stages.

Having found significant effects of Magnesium on spontaneous contractility we next investigated whether these effects were altered when the tissues were stimulated with oxytocin. Using the same protocol as for spontaneous contractions, the data obtained in the presence of oxytocin also showed a concentration-dependent decrease in all parameters of contraction. This was evident in all tissue groups. Representative traces showing the effect of $MgSO_4$ in the presence of oxytocin are given in Figures 3A-D and the mean data are given in Table 2.

Unlike spontaneous activity, in the presence of oxytocin, the myometrium was still producing significant amounts of force at 10mM Magnesium in all preparations. In order therefore to accurately plot concentration-response curves, assist with curve fitting and better understand the response of the tissue, Magnesium was increased to 12 and 20 mM. Typical responses to 20mM Magnesium are shown in Figure 1C.

As is clear in the example traces in Figure 3, for all pregnant tissues, a less potent inhibition with Magnesium was found in the presence of oxytocin compared to its application to spontaneous contractions, i.e. without oxytocin (Figure 2). Additionally, in pregnant tissues, concentration-response curves for Magnesium in the presence of oxytocin were also shifted to the right compared to spontaneous contractions (Figure 3E and 4B-D), resulting in significantly greater IC_{50} values with oxytocin stimulation. The greatest shift in IC_{50} values was seen for term-pregnant tissues from 3.08mM ± 0.16 in spontaneous conditions to 9.75mM ± 0.21 with oxytocin ($P<0.0001$). A significant shift in IC_{50} values was also seen for day 14 gestation tissues from 4.75mM ± 0.21 in spontaneous conditions to 10.25mM ± 0.24 in oxytocin ($P<0.0001$) and day 16 gestation tissues from 5.50mM ± 0.26 in spontaneous conditions to 9.35m ± 0.38 in oxytocin ($P=0.0031$). In contrast, in non-pregnant tissues, the

presence of oxytocin (even at 1nM) did not significantly alter the IC_{50} values for Magnesium: 7.06mM ± 0.32 in spontaneous to 8.07mM ± 0.40 in the presence of oxytocin (Figure 4A, P=0.3236), hence in non-pregnant tissues, Magnesium is equipotent in the presence and absence of oxytocin.

Our data showed that, a consequence of oxytocin significantly increasing the IC_{50} values in all pregnant tissues, was that there were no longer any gestational differences between them, in the effects of Magnesium (Figure 4). In other words, there was no significant differences in the inhibitory effect of Magnesium as pregnancy progressed, unlike what had been found above for spontaneous conditions. Instead, in the presence of oxytocin, the potency of $MgSO_4$ was reduced such that it was similar to its potency in non-pregnant tissues irrespective of pregnancy.

Discussion

Magnesium has been reported to suppress myometrial contractions and for this reason has been used over the last five decades in the treatment of preterm labour. Many clinical studies however, including Cochrane reviews [3], conclude or suggest that $MgSO_4$ is ineffective at delaying labour. Thus, the clinical use of $MgSO_4$ in the treatment of preterm labour is questioned[30-32], highlighting the need for further studies. This study was conducted to better understand the effects of Magnesium on uterine contractility. It was designed to examine if physiological changes, namely gestational state and hormonal background, could influence myometrial responses to Magnesium. In this way, a better understanding of the

disappointing clinical findings will be obtained and perhaps suggest a more stratified approach to its use as a tocolytic, to help prevent preterm labour. Magnesium's effects as a tocolytic may synergise with its use and action to treat eclampsia. Our data provide fresh insights, as they show that preterm myometrium is much less sensitive to the relaxant effects of Magnesium than term myometrium, which could explain its lack of clinical efficacy. If oxytocin was present, its efficacy was further decreased, at all gestational stages. Thus, taken together we conclude that Magnesium's actions are influenced by both gestational state and hormones, such that, at least in mice, it is least effective in early gestation with oxytocin present, and most effective at term in the absence of oxytocin.

In vitro Mouse myometrium

We conducted our study on mouse myometrium so that we could obtain myometrial preparations at several stages of gestation as well as non-pregnant tissue. Myometrium from pregnant mouse has previously been reported to produce rhythmic spontaneous contractions *in vitro* for many hours[21]. Consistent with this, we found all the uterine strips generated spontaneous contractions which were stable within a period of 45 -60 minutes, and remained regular without significant reduction in amplitude or frequency for many hours. This therefore allowed the effect of incremental concentrations of Magnesium to be examined and concentration-response curves to be fitted. There has been just one previous report of Magnesium's actions on mouse myometrium, in a lipopolysaccharide model of preterm birth[27]. These authors only studied mid-gestation (14 day) uterus and reported inhibition of spontaneous contractions with Magnesium; an effect which was decreased by lipopolysaccharide. Our findings of an inhibitory effect on term-and non-pregnant myometrium are consistent with findings in other species[10,12,14,15,33] including humans. Future

studies should attempt to obtain human biopsies at different stages of gestation to confirm these findings ~~herein~~, although obtaining preterm biopsies is challenging. In addition, to reflect the somewhat heightened state of contraction that may be associated with preterm labor compared to the non-laboring strips used here, a preterm labor mouse model, such as that induced by LPS or other agents, c~~sh~~ould be used to investigate the *in vivo* therapeutic effect of $MgSO_4$.

Magnesium's mechanism of action in myometrium.

The effect of $MgSO_4$ on contractions at different gestational stages was investigated and our data shows that regardless of gestational age or pregnancy status, it can reduce myometrial contractions. This inhibitory effect was reversible and the prompt recovery after washout suggests no ill effects of Magnesium on myometrium, even at high concentrations. The concentrations of $MgSO_4$ used by us *in vitro* were empirically determined, and in the case of oxytocin-evoked contractions, going in to pharmacological rather than physiological concentrations, to enable maximal effects to be obtained.

It is unlikely that intracellular Magnesium will have risen during the course of the experimental protocol, due to the activity of Na^+ - Mg^{2+} exchangers and intracellular buffers[34], as well as Magnesium's slow penetrability through cell membranes [35]. That the effects of Magnesium on the myometrium are relatively rapid and reversible, suggests that its main mechanism of action is likely to be extracellular. The rapid reversal of Magnesium's effect is clinically useful, for example if delivery is by cesarean section, as it reduces the risk of postpartum haemorrhage [36]. Magnesium use in preeclampsia has been associated with increased post-partum haemorrhage in some studies [37], but in another study, its use was not associated with additional blood loss [38].

The major mechanism of Magnesium's action to relax uterine smooth muscle is, as with other excitable tissues, due to its cationic competition with Calcium [15]. In myometrium, contractions, whether spontaneous or agonist induced, are critically dependent upon Calcium entry through L-type Calcium channels[39,40]. Thus when increased Magnesium competes with Calcium, entry of Calcium falls and hence contractions reduce and can even be abolished, as we and others have shown [13]. The fall in intracellular Calcium will also lead to a fall in the Calcium content of the sarcoplasmic reticulum (SR)[41]. This reduced SR Calcium available when agonists such as oxytocin produce IP_3, will reduce their ability, to increase myometrial contractility[15]. In this way, Magnesium will be expected to reduce the force of spontaneous and oxytocin-induced contractions. Fomin et al., (2006) showed that $MgSO_4$ reduced spontaneous, oxytocin- and KCl-induced myometrial contractility and all were associated with a decrease in intracellular Calcium[34]. In addition, they found no shift in the force-Ca relationship in the myometrium. Together this provides strong evidence that Magnesium's effects are predominantly extracellular and on Calcium entry.

At all gestations and in non-pregnant tissue, our data show a significant reduction in force and frequency of contractions with addition of $MgSO_4$. A reduction in force is most likely because of Magnesium's antagonistic effect with Calcium at L-type calcium channels, as discussed above. Frequency of contractions is mainly dependent on excitability and membrane potential, hence a reduction in frequency suggests the pacemaker activity may also be affected by $MgSO_4$ [11]. For example, should differences in the resting membrane potential of some pacemaker cells exist, it may make them more susceptible to the effects of Magnesium leading to fewer action potentials being triggered [42]. Our figures throughout show that, a decrease in the frequency of contractions usually preceded a fall in force. This

finding is consistent with observations on human myometrium[12,43]. Additionally, although not yet tested in uterus, Magnesium may affect inter-cellular coupling such as via gap junctions [44,45], hence reducing the likelihood of frequent, synchronous contractions being produced.

Gestational effects

In spontaneous contractions, the effect of $MgSO_4$ was most potent at term, and least potent in non-pregnant tissues. The latter group were not staged for estrous and should therefore be treated as a mean of any cyclical, estrous changes[46,47] We found that even on day 16 of gestation, Magnesium's effect on the myometrium was significantly less than at term, suggesting that the changes in sensitivity are on-going throughout pregnancy. Given that the underlying mechanism of Magnesium's effects is via decreasing excitability and Calcium entry, then our findings point to a difference in the Calcium channels with gestation. There have been surprisingly few studies of L-type Calcium expression with gestation in the myometrium. The work that has been done suggests an increase in expression and changes of subunits from mid-gestation onwards. Tezuka et al (1995)[16] found a marked increase in α1 expression in pregnancy, especially in the last half of gestation until term, followed by a decrease during labour. Similarly, Mershon *et al* (1994)[17] studied the expression of α_1 subunit and showed a gradual increase in the mRNA towards term followed by a decrease during parturition. They also showed an increase in the number of dihydropyridine binding sites (markers for L-type channels) in the last half of gestation. Thus, a simple effect on Calcium entry is difficult to propose to explain gestational differences, unless the switch to increased α-1 subunits confers an increased susceptibility to Magnesium blockade. Rather, our data suggests that at

term the effect of Magnesium is ~~sufficient~~enough to reduce excitability and make action potential firing less likely, as occurs with its use in neuronal tissues to protect the brain.

Oxytocin and Magnesium

To determine if $MgSO_4$ can affect contractile parameters in the presence of an agonist, its effect on oxytocin-evoked contractions was determined. Oxytocin was used due to its particular importance to labour. Oxytocin can directly and indirectly stimulate myometrial contractions [19]. Our data show that a greater concentration of Magnesium is needed to inhibit oxytocin-stimulated contractions compared to spontaneous contractions in pregnant tissues. This is in agreement with data from other studies including human myometrium [14,33]. However, oxytocin did not significantly change the IC_{50} for Magnesium in non-pregnant tissues, from that found for spontaneous contractions. We found this to be the case even when oxytocin was used at 1nM i.e. double the oxytocin concentration used in pregnant tissues. There was no difference in the potency of $MgSO_4$ on oxytocin-stimulated contractions between the different gestational groups. The greatest shift in Magnesium's potency with oxytocin was observed for term-pregnant myometrium; this shifted the IC_{50} from around 3 mM in spontaneous conditions to almost 10 mM. In terms of clinical applications, the effect of oxytocin is to shift the dose from therapeutic to lethal. Therapeutic doses of Magnesium are reported at 2.5 mM (5 mEq/L) or below [12], and for seizure management in preeclampsia, 3 mM [33].

Although oxytocin acts via several mechanisms, an important mechanism is increasing membrane potential (resulting in opening of the L-type Ca^{2+} channels and increased Calcium entry), releasing Calcium from the sarcoplasmic reticulum and preventing Calcium exit [19]. These mechanisms will counteract Magnesium's actions and may explain why a greater

concentration of $MgSO_4$ is needed to inhibit oxytocin induced contractions close to term. The lipid environment around the oxytocin receptor influences its affinity for oxytocin [19,48,49], an effect attributed to its partitioning into lipid rafts and their effect on signal transduction [50,51]. It was also reported that as well as high cholesterol, the high affinity form of the oxytocin receptor requires Magnesium, working as an allosteric modulator. Thus, when Magnesium is increased it may increase the oxytocin signalling and further counter the relaxant effect of Magnesium on Calcium entry.

Transition to labour in humans is associated with increased oxytocin receptor expression [52], which increases the sensitivity of the uterus towards oxytocin [53]. An increase in oxytocin receptor expression towards term in mouse, has also been shown [53]. Consistent with this is our finding that little, if any, contractility stimulation to 0.5nM oxytocin was observed in non-pregnant tissues and hence the use of 1nM, to assess the effect of oxytocin on the potency of $MgSO_4$ (control data, not shown). Although not accounting for the lack of gestational differences, the increased drive on contraction produced by oxytocin, and the increased number of receptors, and increased affinity, may also partly explain why a greater concentration of $MgSO_4$ is needed to inhibit -contractions in its presence. In addition, other mechanisms operated by Calcium-independent pathways may also be stimulated by oxytocin and be affected by elevated Magnesium. However, the role of Ca-sensitisation in uterus is thought to be minor [54]. This work, and studies by others, has not considered possible effects of magnesium on potassium channels.

Conclusion

This *in vitro* study shows that $MgSO_4$, acting extracellularly, concentration-dependently inhibits spontaneous and oxytocin-induced myometrial contractions in both the pregnant and non-pregnant mouse, with greater efficacy observed in term-pregnant tissues. However, oxytocin decreases the potency of $MgSO_4$ in pregnant tissues, due to its stimulation of contraction and perhaps due to Magnesium allosterically increasing the affinity of the oxytocin receptor, which may underlie its lack of efficacy as a relaxant i.e. tocolytic, *in vivo*.

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Conflicts of Interest

None

Author Contributions.

SA and SW conceived the study. BO, SA & SW designed protocols, BO conducted the experimental work, BO & SA undertook the analysis, BO, SA & SW drafted, revised and agreed the submitted manuscript.

Table 1:

Changes in contractile properties of spontaneous contractions in response $MgSO_4$

Contractile properties	Gestational States	2mM (%±S.E.M)	4mM (%±S.E.M)	6mM (%±S.E.M)	8mM (%±S.E.M)	10mM (%±S.E.M)
Amplitude	Non-pregnant	95.8 ± 1.1 *	93.1 ± 1.6 *	90.9 ± 2.3 **	86.2 ± 3.1 **	81.5 ± 3.5 **
	Mid-term	94.6 ± 1.6	90.6 ± 2.4	83.9 ± 3.1 **	65.8 ± 11.6 *	57.3 ± 14.9 **
	Term-pregnant	91.8 ± 2.7 *	61.8 ± 10.9 **	56.9 ± 11.5 **	38.3 ± 9.9 **	19.6 ± 9.8 ***
Frequency	Non-pregnant	87.9 ± 4.9 *	70.6 ± 5.0 **	62.7 ± 4.3 **	45.9 ± 5.7 **	31.2 ± 2.9 **
	Mid-term	84.9 ± 8.7	64.9 ± 7.0 **	48.9 ± 3.9 **	32.2 ± 9.01 **	23.4 ± 8.3 **
	Term-pregnant	72.5 ± 6.7 **	43.6 ± 8.2 **	35.5 ± 7.8 ***	20.5 ± 7.1 **	9.6 ± 5.0 ***
AUC	Non-pregnant	83.7 ± 4.4 **	73.1 ± 4.2 **	57.7 ± 5.3 **	48 ± 5.1 ***	40.1 ± 4.5 ***
	Mid-term	83.9 ± 4.7 *	63.7 ± 4.2 *	48.0 ± 5.1 **	26.9 ± 10.5 **	19.9 ± 9.4 **
	Term-pregnant	71.3 ± 6.4 **	43.3 ± 6.7 ***	33.1 ± 6.5 ***	18.0 ± 5.4 ***	6.1 ± 3.2 ***

Mean data showing concentration-dependent decrease in amplitude, frequency and AUC of spontaneously contracting non-pregnant, mid-term (day 14 and 16) and term-pregnant myometrium. Values are expresses as Mean ± standard error mean (S.E.M). A significant difference in activity was found using ANOVA with Bonferroni post hoc analysis. * denotes a significant difference compared to control period (100%) $p<0.05$, $**p<0.01$, $***p<0.0001$.

Table 2:

Changes in contractile properties of oxytocin-induced contractions in response to $MgSO_4$

Contractile properties	Gestational States	2mM (%±S.E.M)	4mM (%±S.E.M)	6mM (%±S.E.M)	8mM (%±S.E.M)	10mM (%±S.E.M)	12mM (%±S.E.M)	20mM (%±S.E.M)
Amplitude	Non-pregnant	98.7 ± 0.7	96.7± 1.1 *	95.0 ± 2.1 *	92.7 ± 2.4 *	90.8 ± 2.6 **	89.3 ± 1.9 *	81.2 ± 5.4*
	Mid-term	98.1 ± 1.2 *	90.5 ± 4.8 *	84.2 ± 3.7 **	81.2 ± 4.8 **	72.2 ± 8.1**	62.8 ± 5.4 ***	5.9 ± 2.6**
	Term-pregnant	98.8 ± 1.0 *	95.5 ± 1.2 **	90.6 ± 1.7 **	84.0 ± 2.0 **	71.3 ± 7.4 ***	71.0 ± 4.9 ***	4.4 ± 4.4**
Frequency	Non-pregnant	85.3 ± 2.7 **	66.1 ± 4.0 **	48.0 ± 5.0 **	40.0 ± 5.6 **	35.7 ± 5.5 **	36.1 ± 4.1 ***	47.0 ± 7.9**
	Mid-term	92.8 ± 3.1 *	90.4 ± 4.1 *	79.9 ± 11.8 **	75.4 ± 11.9 **	67.5 ± 10.5 **	58.0 ± 13.2 **	2.5 ± 0.1
	Term-pregnant	93.9 ± 2.3	88.3 ± 3.2 **	77.2 ± 3.2 **	65.7 ± 3.4***	57.9 ± 3.3 ***	58.0 ± 3.9 ***	1.8 ± 1.8***
AUC	Non-pregnant	88.7 ± 2.6 **	73.4 ± 4.9 **	58.0 ± 6.1 **	54.2 ± 5.0 **	44.8 ± 5.21 **	38.9 ± 0.9 **	38.8 ± 5.8**
	Mid-term	94.7 ± 2.5 *	90.0 ± 3.3 *	67.1 ± 11.0 **	65.6 ± 4.5 ***	48.8 ± 9.3 **	34.9 ± 8.8**	2.1 ± 0.9***
	Term-pregnant	93.8 ± 1.2	83.3 ± 1.5 **	73.6 ± 2.6 **	60.9 ± 3.5 **	50.5 ± 3.9 **	41.3 ± 6.7 **	1.7 ± 1.7***

Mean data showing concentration-dependent decrease in amplitude, frequency and AUC of oxytocin-induced contracting non-pregnant, mid-term (day 14 and 16) and term-pregnant myometrium. Values are expressed as Mean ± standard error mean (S.E.M). Significant difference in activity was found using ANOVA with Bonferroni post hoc analysis. * denotes a significant difference compared to control period (100%) $p<0.05$, **$p<0.01$, ***$p<0.0001$.

Figure legends

Figure 1: The effects of Magnesium on contractions of mouse myometrium.

Typical records showing contractions obtained from term-pregnant mouse myometrium. In this and all subsequent figures, data were obtained at 37˚C and pH 7.4, with tissues superfused with physiological saline (containing 1. 2 mM $MgSO_4$), in the absence (**A** & **B**) and presence of increased (10 or 20 mM) Magnesium as sulfate (**C**) or chloride (**D**) salt. Traces B, C & D, were in the presence of oxytocin (0.5 nM).

Figure 2: The effects of Magnesium sulfate on spontaneous contractions of mouse myometrium.

Representative isometric traces showing the effect of increasing concentrations of Magnesium sulfate in **(A)** non-pregnant, **(B)** 14-day pregnant, **(C)**, 16-day pregnant and (**D)** term-pregnant myometrium. The coloured bars indicate the 15-minute period when Magnesium sulfate was added. In all gestational states, a reduction in amplitude and frequency was observed with increased concentration**. (E)** The concentration-response curves showing the effect of Magnesium sulfate on force area under the curve (AUC) at different gestational states (non-pregnant : green circle, 14-day: blue square, 16-day: red diamond, term pregnant: purple triangle). The concentration-response curves significantly shifted to the left as gestation increased (P<.0001). Significant difference was found using ANOVA with Bonferroni post hoc test.

Figure 3: The effects of Magnesium sulfate on oxytocin-induced contractions of mouse myometrium.

Representative isometric traces showing the effect of increasing concentrations of Magnesium in **(A)**, non-pregnant myometrium, **(B)**, 14-day pregnant, **(C)**, 16-day pregnant and **(D),** term-pregnant myometrium in the presence of oxytocin (1 nM used for non-pregnant tissue and 0.5 nM for all pregnant tissues). The short, coloured bars indicate the 15-minute period where Magnesium sulfate was added. Increasing concentration of Magnesium sulfate (2-12mM) caused gradual reduction in amplitude and frequency of contractions in all gestational states. **(E)** The concentration-response curves show the effect of Magnesium sulfate on AUC of contraction at different gestational states (non-pregnant: green circle, 14-day: blue square, 16-day: red diamond, term-pregnant: purple triangle). There was no significant difference between the concentration-response curves for the different gestational states, determined using ANOVA with Bonferroni post hoc test.

Figure 4: The effect of Magnesium sulfate between spontaneous and oxytocin-induced contractions at different gestational states.

The concentration-response curves show the effect of Magnesium sulfate on force integral (AUC) of contraction in spontaneous (green circles) and oxytocin-induced contractions (purple squares). There was no significant difference (P=0.062) between the concentration-response curves of non-pregnant myometrium **(A)**. For 14-day **(B)**, 16-day **(C)** and term-pregnant myometrium **(D)**, the concentration-response curves were significantly shifted to the right in the presence of oxytocin, indicating a significantly greater concentration of Magnesium is required to reduce contractions in the presence of oxytocin compared to spontaneous contractions (P<.0001, P<0.01 and P<0.0001 respectively). Significant difference in activity was determined using the extra sum of squares F-test.

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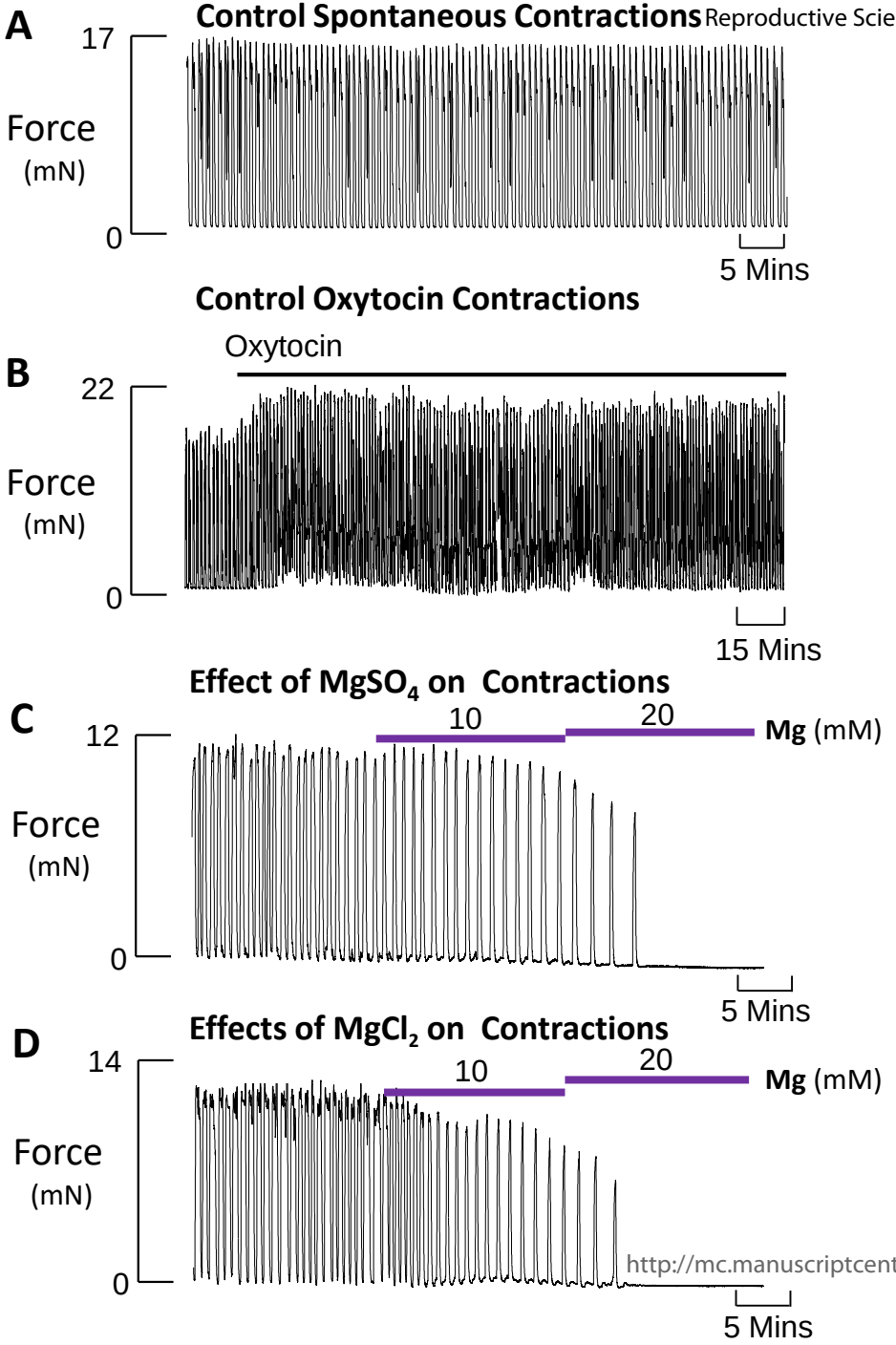
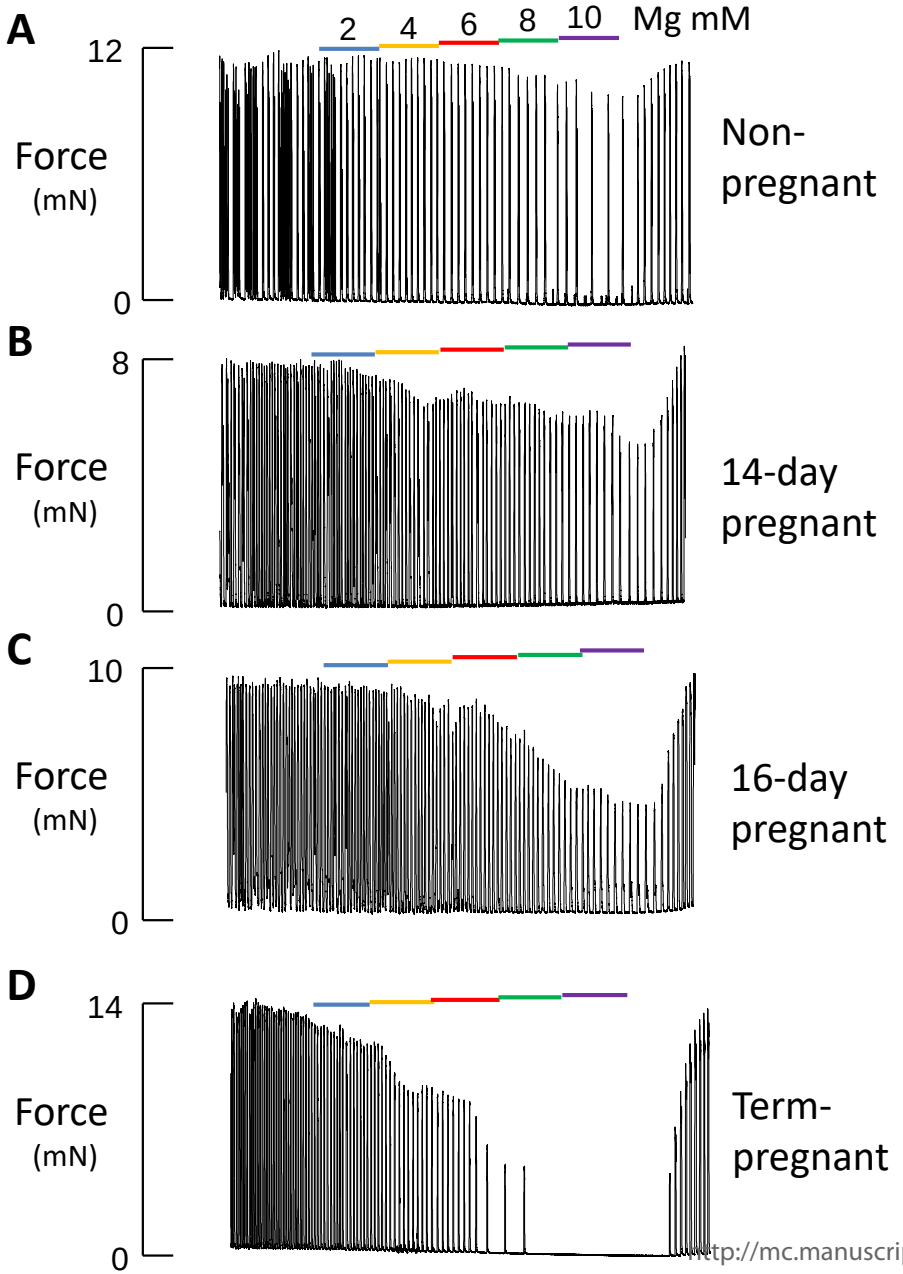


Figure 1: The effects of magnesium on contractions of mouse myometrium.

Typical records showing contractions obtained from term-pregnant mouse myometrium. In this and all subsequent figures, data were obtained at 37˚C and pH 7.4, with tissues superfused with physiological saline (containing 1. 2 mM $MgSO_4$), in the absence (**A** & **B**) and presence of increased (10 or 20 mM) magnesium as sulfate (**C**) or chloride (**D**) salt. Traces B, C & D, were in the presence of oxytocin (0.5 nM).

Magnesium on spontaneous contractions



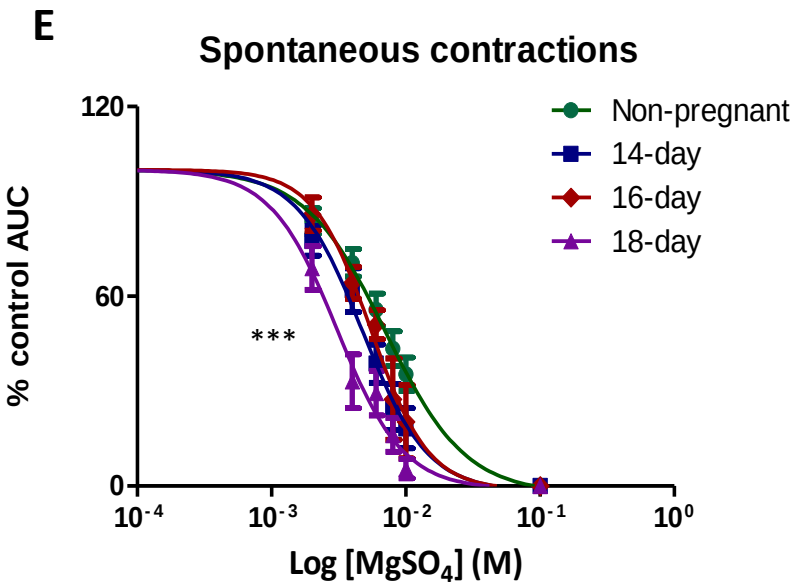
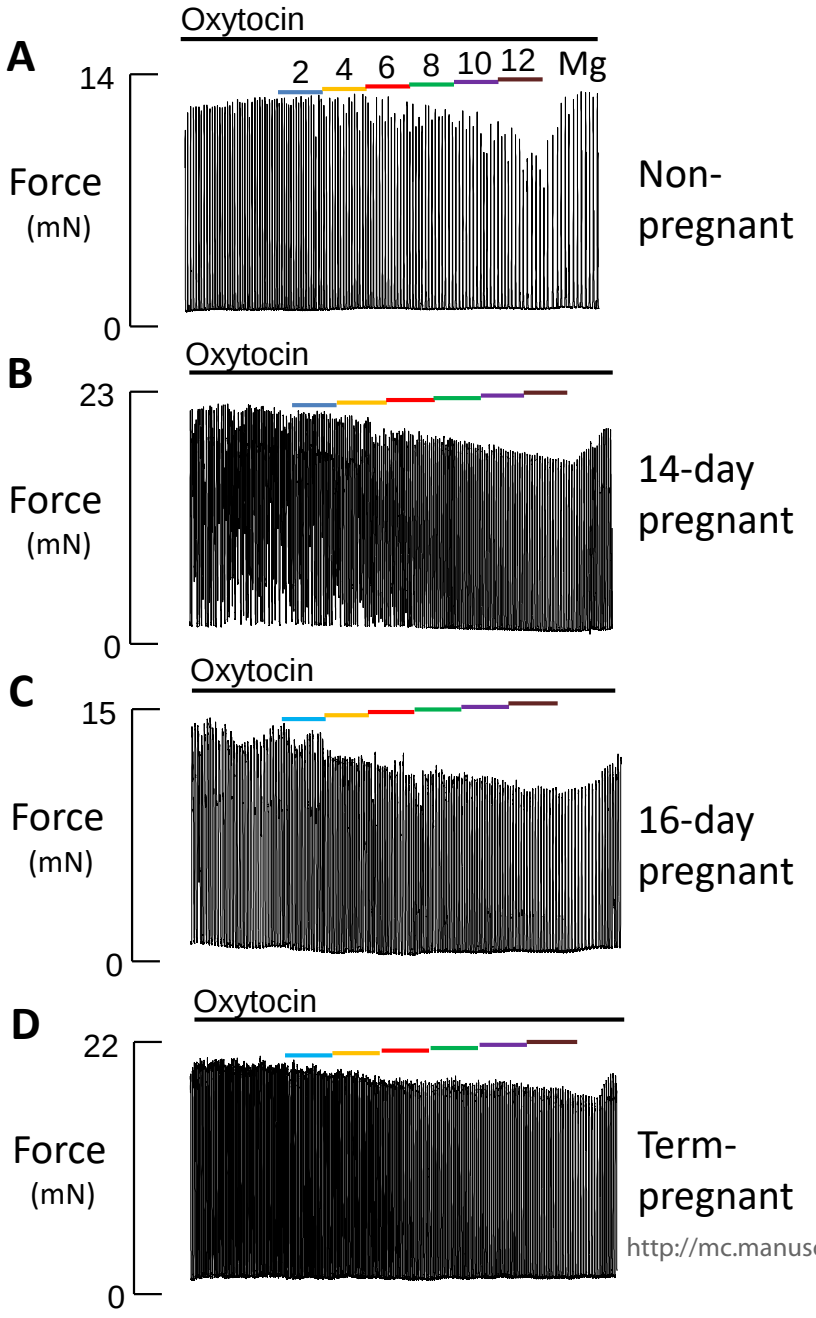


Figure 2: The effects of magnesium sulfate on spontaneous contractions of mouse myometrium. Representative isometric traces showing the effect of increasing concentrations of magnesium sulfate in **(A)** non-pregnant, **(B)** 14-day pregnant, **(C)**, 16-day pregnant and (**D)** term-pregnant myometrium. The coloured bars indicate the 15-minute period when magnesium sulfate was added. In all gestational states, a reduction in amplitude and frequency was observed with increased concentration**. (E)**The concentration-response curves showing the effect of magnesium sulfate on force area under the curve (AUC) at different gestational states (non-pregnant : green circle, 14-day: blue square, 16-day: red diamond, term pregnant: purple triangle). The concentration-response curves significantly shifted to the left as gestation increased (P<.0001). Significant difference was found using ANOVA with Bonferroni post hoc test.

Magnesium on Oxytocin-induced contractions



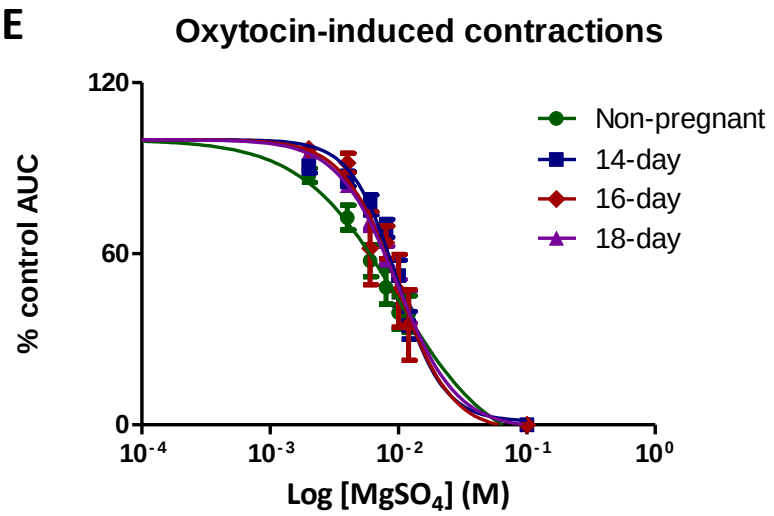


Figure 3: The effects of magnesium sulfate on oxytocin-induced contractions of mouse myometrium.

Representative isometric traces showing the effect of increasing concentrations of magnesium in **(A)**, non-pregnant myometrium, **(B)**, 14-day pregnant, **(C)**, 16-day pregnant and **(D),** term-pregnant myometrium in the presence of oxytocin (1 nM used for non-pregnant tissue and 0.5 nM for all pregnant tissues).The short, coloured bars indicate the 15-minute period where magnesium sulfate was added. Increasing concentration of magnesium sulfate (2-12mM) caused gradual reduction in amplitude and frequency of contractions in all gestational states. **(E)** The concentration-response curves show the effect of magnesium sulfate on AUC of contraction at different gestational states (non-pregnant : green circle, 14-day: blue square, 16-day: red diamond, term-pregnant: purple triangle). There was no significant difference between the concentration-response curves for the different gestational states, determined using ANOVA with Bonferroni post hoc test.

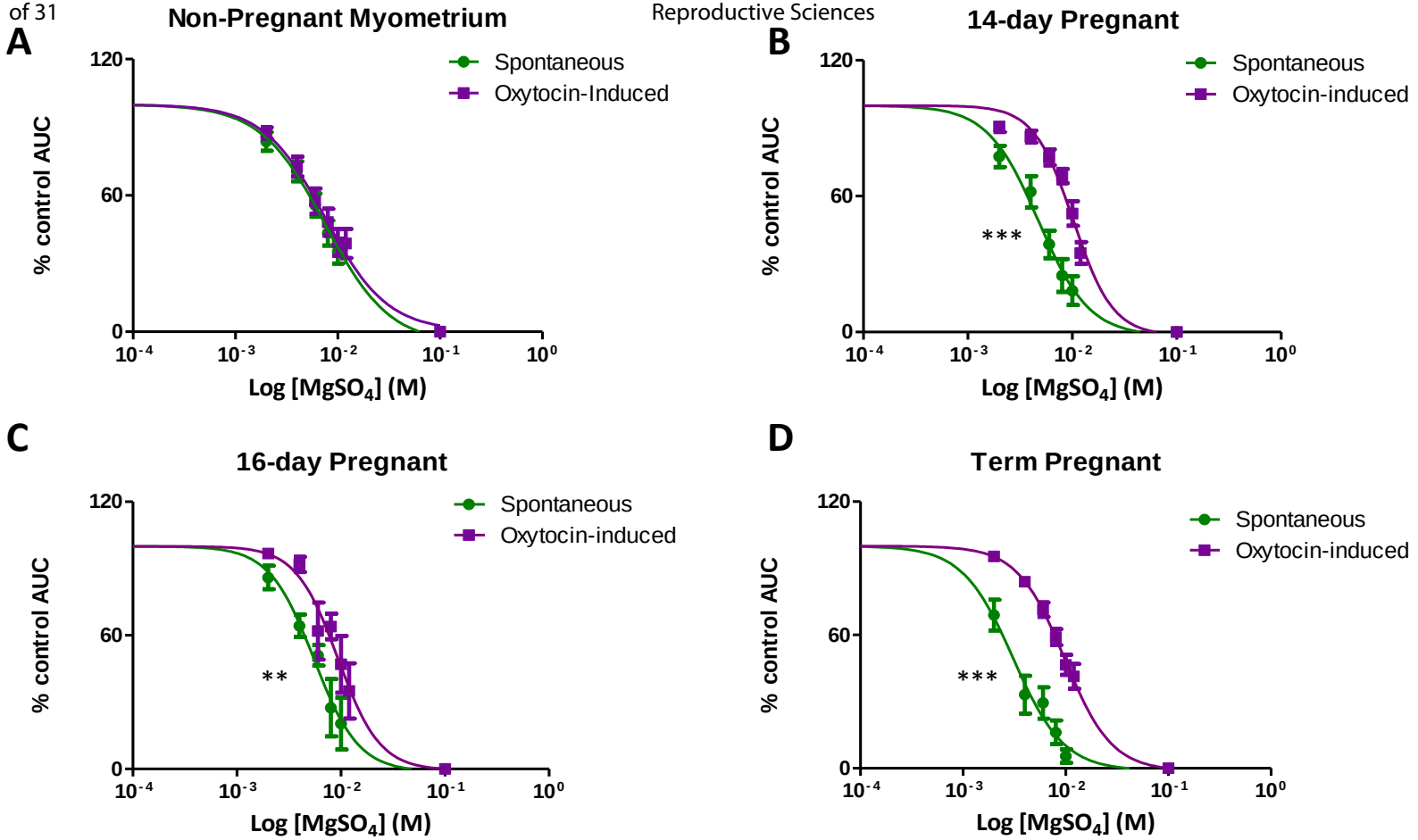


Figure 4: The effect of magnesium sulfate between spontaneous and oxytocin-induced contractions at different gestational states.

The concentration-response curves show the effect of magnesium sulfate on force integral (AUC) of contraction in spontaneous (green circles) and oxytocin-induced contractions (purple squares). There was no significant difference (*P*=0.062) between the concentration-response curves of non-pregnant myometrium **(A)**. For 14-day **(B)**, 16-day **(C)** and term-pregnant myometrium **(D)**, the concentration-response curves were significantly shifted to the right in the presence of oxytocin, indicating a significantly greater concentration of Mg is required to reduce contractions in the presence of oxytocin compared to spontaneous contractions (** indicates *P*<0.01, ***P*<0.0001). Significant difference in activity was determined using the extra sum of squares F-test.